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14. ABSTRACT **Approx 200 words** unclassified summary of the most significant finding during the research period
Enhanced tumorigenicity of the PMC42-LA cell line after transfection with a library of shRNAmir constructs has been validated in a repeat experiment, and replicate material obtained for next generation sequencing. The methodology for the sequencing has been revised and re-validated, causing some delays, however it is now imminent. It will be possible, using the revised methodology, to sequence the shRNA associated with both altered colony morphology and EpCAM expression *in vitro*, as well as the formation of tumors *in vivo*. The in vivo relevance of EpCAM levels has been affirmed. Extension of the boutique library of 4,462 shRNA constructs to a full genome screen in the PMC42-LA cell line will not proceed due to the time required for this model. Instead, more emphasis will be placed on the MDA-MB-468 model where we have extensively characterized the in vivo EMP (two distinct zones at the tumour periphery and stromal interface, respectively), as well as circulating tumor cells and lung micrometastases. Again, metastasis from this model has taken longer than anticipated, resulting in additional delays. Nonetheless, these cells have been tagged with Luciferase2, and will be subjected to *in vivo* testing after transfection with a full genome library of shRNAmir constructs. The 'reporter assays' required for Aim three have been developed and await the completion of Aims 1 and 2, however, they have already been successfully used in additional screens outside of this grant.

15. SUBJECT TERMS
Key words or phrases identifying major concepts in the report: Epithelial Mesenchymal Transition (EMT), functional genomics, micrometastasis, macrometastasis, shRNA

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Introduction

We hypothesize that epithelial – mesenchymal phenotypic attributes affect the capacity of single cells to establish a macroscopically detectable cancer mass, and thus play an etiological role in tumorigenicity, invasion, metastasis and recovery after seemingly effective chemotherapy of breast cancer cells. This will increase our understanding of the role of EMT in breast cancer, provide novel reagents and tools for the study of breast cancer, and provide new leads for therapeutic targeting in breast cancer. We are employing the high throughput functional genomic screens using epithelial mesenchymal transition (EMT)-capable PMC42 and MDA-MB-468 human breast cancer cell lines to identify molecular factors controlling these processes, and test their relationship to the EMT process. To date we have progressed the project with the PMC42-LA cells to the extent that tumors have been found after ~ 7 months compared to minimal growth of the parental cells. Parallel analysis *in vitro* has shown morphological changes and altered profile of cell surface EpCAM, which we have independently validated to associate with decreased EMT. The overall project is behind schedule due to unanticipated delays beyond our control, and a 12 month 'no-cost extension' has been applied for. Nonetheless, the project remains viable and exciting, with our first round of sequencing from in vitro and in vivo hits in stream. We anticipate completion in the coming year.

Body

Task 1: To identify gene products which may constitutively block the growth of PMC42 human breast cancer cells in SCID mice

We have essentially completed the core deliverables for tasks 1a to 1f. Some minor modifications to the experimental approach were necessary, as detailed in previous annual (2011) report. We have been successful in initiating tumorigenicity for the human PMC42 breast

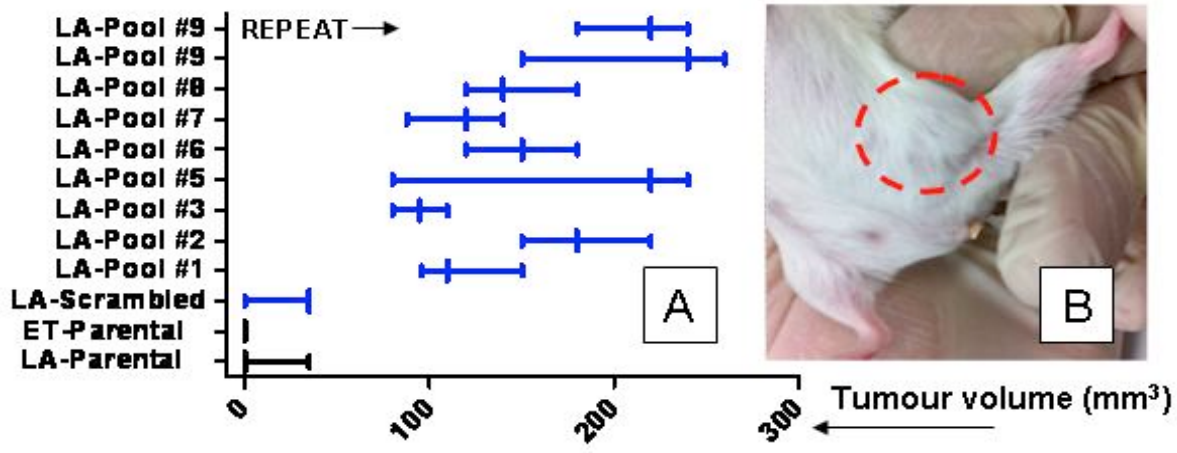

Figure 1: **A** tumour volumes from boutique shRNA library transfections into PMC42-LA following 6 months growth. Parental lines and scrambled control show negligible growth while all other transfected pools displayed tumorigenicity. **B** Boutique pool #9 at 7 months.

tumor cells after shRNA library transduction, however palpable tumors take 4 to 6 months to develop in SCID mice (Figure 1). This has caused significant delays in our original timelines. Despite this we are achieving our aims and tumors are or sufficient quality for IHC and genomic analysis (Figure 2). So far, a number of shRNA species have now been identified from boutique library and additional PMC42 tumor samples are currently being analyzed by NextGen sequencing protocols as indicated below. We will not pursue further shRNA library screening in the PMC42 model due to their very slow growth properties, as we have significant number of 'hits' from the described boutique screen.

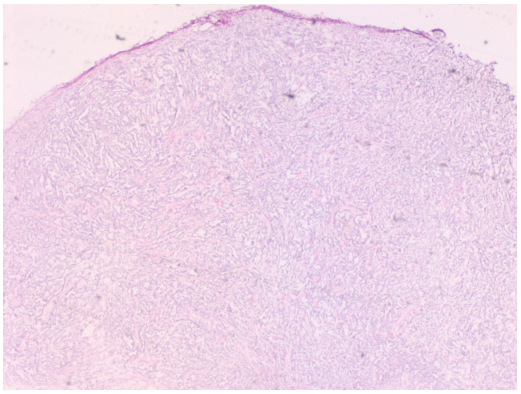

Figure 2: Representative boutique pool PMC42-LA tumours (H&E staining) indicate moderate- to well-differentiated adenocarcinoma.

During the no cost extension through to October 2013 we plan:

- Multiplex NextGen sequencing of additional tumor samples to complete an inventory of shRNA hits. These will complement an orthogonal list generated from Task 2.

- Alhough outside the initial proposal, we will investigate the transcriptome profile of generated samples for potential identification of tumor initiation signatures. This will be analysed in the context of genome wide-profiling of these cells for in vitro EMT caused by EGF, or in comparison between the LA (epithelial subline) and ET (mesenchymal parental) cells.

- General histological analysis has been performed on the shRNA-induced PMC42 tumors. We will extend this to look for evidence of EMT in these primary tumor samples. This will be accomplished by IHC for Vimentin (Vim) and E-Cadherin, with a particular focus on Vim induction at the invasive edge of formed tumors generated by shRNA transduction.

Task 2: To identify gene products that may constitutively block the spontaneous capacity of MDA-MB-468 human breast cancer cells to form metastases in SCID mice

Human MDA-MB-468 cells undergo EMT at the xenograft edge and liberate CTCs, but form micrometastases at low frequency. They do not form overt lesions when inoculated intracardially. Under this grant, we have been successful in Luciferase tagging these cells (pGL4.50 [luc2/CMV/Hygro), and completed extensive preliminary experiments in mice to reconfirm the metastasis frequency (Figure 3). As with the PMC42 cells, these xenografts are also very slow growing (~4 to 6 months), and this too has led to a delay in our progress. We have pre-titrated the shRNA library and determined the MOI for these cells. During the no cost extension through to October 2013 we plan to:

- Transduction of luciferase-tagged MDA-MB-468 with a full genome wide shRNA library, followed by expansion and enrichment of transduced cells. That is, generate stocks of luciferase-tagged MDA-MB-468 cells, transduced with a library comprising ~ 100,000 clones.

- Graft boutique shRNA transduced MDA-MB-468 cells into SCID mice and isolate macrometastic lesions (focusing predominantly on lung and marrow). Collected material will be examined for identification of shRNAs as described under Task1.
- We will not evaluate the boutique shRNA library for the MDA-MB-468, but instead use the complete library – which encompasses the boutique (~3,600 shRNA) set.

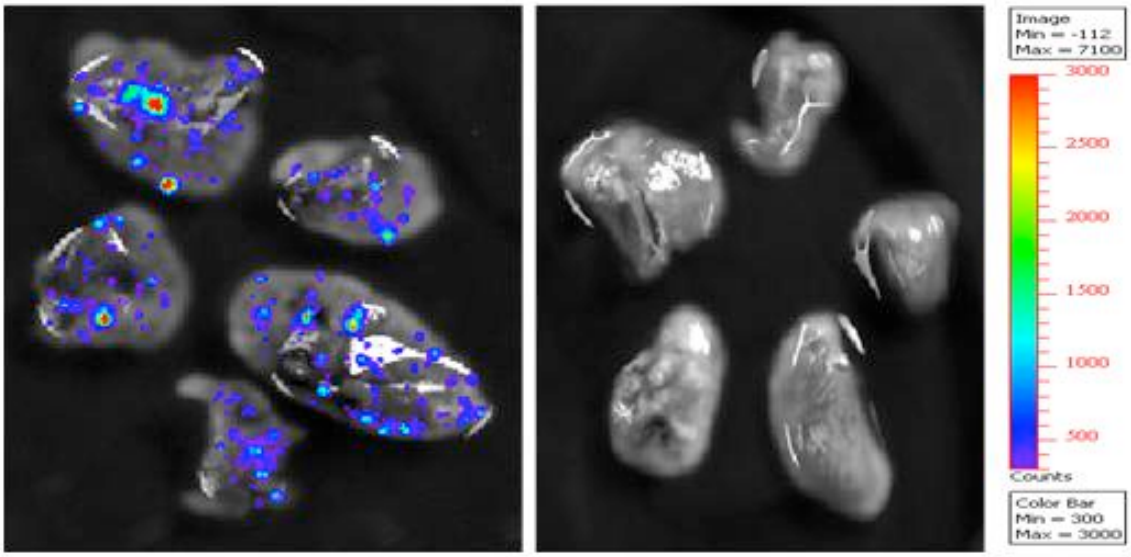

Figure 3: MDA-MB-468-luc2/CMV/Hygro cells have been grafted *in vivo* and validated to have similar primary growth and metastatic properties to parental cells. Tumors grow slowly at the primary site and metastasisze to soft organs where they reside as single dormant cells. Over time, a very low proportion of these grow to macrometastases. These properties and the ability to monitor this process is a particularly desirable for the final phase of the work. Depicted on left is Luc2 detected *ex vivo* (IVIS bioluminescence) for lungs taken from mice where the luc2-tagged cells have grown over a 6 month period. H&E staining of these lungs detect one or two macrometastases; other single focal points appear to be single cells. Panel on the right are control lungs from a non-tumor challenged mouse.

Task 3: To identify and characterize novel gene products elucidated in Aims 1 and 2 that enact a switch between epithelial and mesenchymal states

Under this Aim, candidate shRNAs identified in Aims 1 and 2 will be assembled individually or assembled into a smaller, dedicated screen and tested for their ability to influence EMT or MET. Novel candidates confirmed to play a role in EMT will be tested fully with *in vitro* and *in vivo* analysis. Thus far we have completed core objectives for Tasks 3 a–c.

With respect to Task b, and as reported previously, mesenchymal colonies were observed in varying proportions when the PMC42-LA cells transduced with the boutique shRNA library sub-pools were plated at low density. These analyses are additional to those originally proposed but

are consistent with the *in vivo* approach and analysis of the *in vitro* phenotype is being carried out in parallel. FACS analysis of wild type and library sub-pool transduced PMC42-LA cells for the epithelial marker EpCAM revealed that sub-pool #5, which exhibits the lowest proportion of

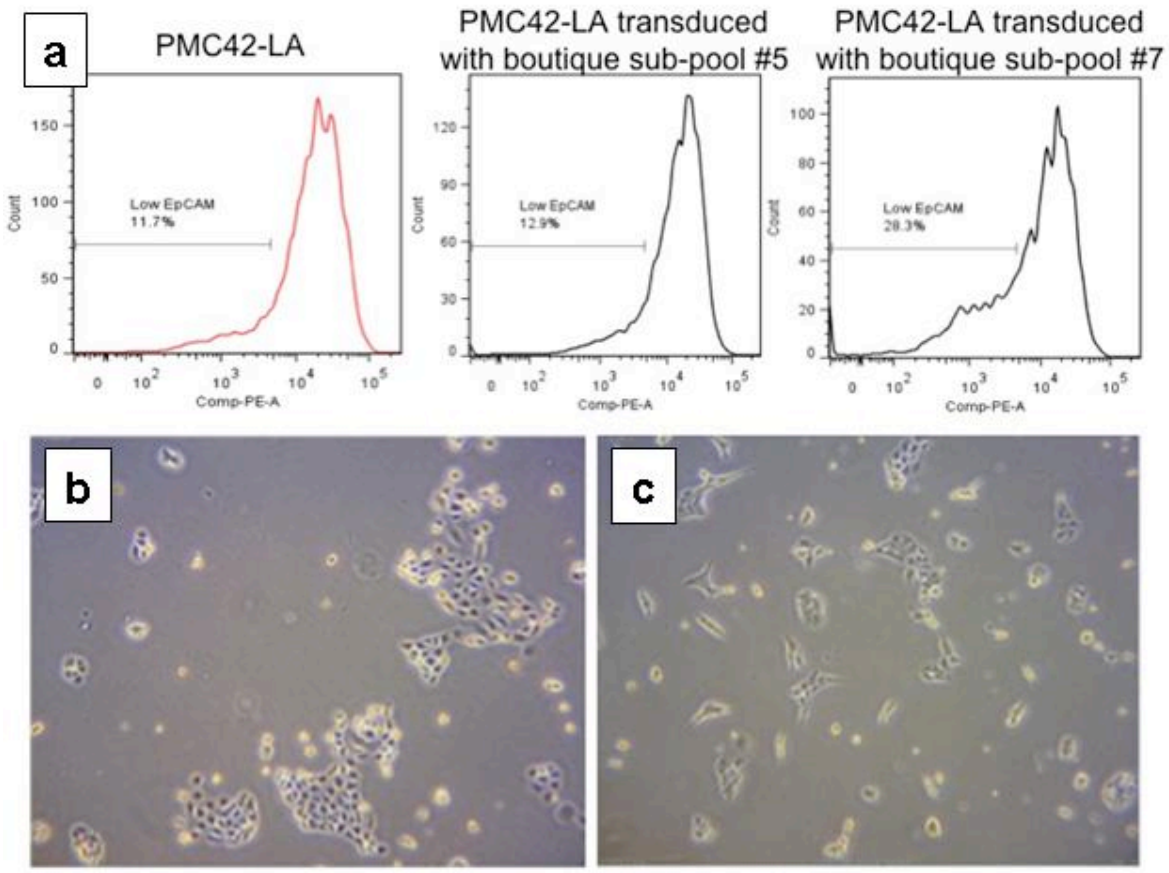

Figure 4: (a) FACS analysis of EpCAM surface expression in shRNA library untransduced PMC42-LA cells and selected boutique subpools. (b & c) Culture morphology of PMC42-LA cells selected by FACS for high (b) and low (c) surface expression of EpCAM.

mesenchymal colonies amongst the sub-pools, had a similar EpCAM profile to untransduced PMC42-LA cells, while sub-pool #7, which exhibits the largest proportion of mesenchymal colonies, had a markedly increased proportion of cells with low EpCAM expression (Figure 4).

Although tumors grow very slowly, EpCAM-high and EpCAM-low populations have been sorted from the transduced PMC42-LA cells and grafted into the mammary fat pad of SCID mice. Tumorigenicity, epithelial-mesenchymal modulation, and (eventually) metastasis endpoints will be evaluated in these mice. During our no cost extension through to October 2013 we plan:

- Further prioritization of EMT/MET/BCSC gene hits for this study, and dissemination of non-EMT/MET/BCSC to the research community.

- *In vitro* analysis of up to 10 EMT/MET perturbational candidates. For this, characterization of the *in vitro* proliferative, migratory, and invasion-regulating potential of candidates, analysis of EMT perturbational mechanism, analysis of relationship to BCSC phenotype.

- *In vivo* analysis of up to 3 EMT/MET perturbational candidates for effects on tumorigenic potential of PMC42-LA and macrometastatic potential of MDA-MB-468; analysis of molecular consequences and morphologic effects *in vivo*.

Key Research Accomplishments

- Orthotopic tumors have been grown from PMC42-LA cells transduced with a boutique library, and sub-pools thereof, consisting of shRNA targets selected as markers and mediators of EMT, metastasis, migration, and breast cancer stem cells. Tumors have been analyzed and shRNA species identified that enable tumorigenicity. Multiplex NextGen sequencing of additional tumor samples are ongoing to complete an inventory of shRNA hits. The enhanced tumorigenicity of selected subpools has been verified in a second experiment, against vector controls.

- As reported last year, responsive subpopulations have been detected in both MDA-MB-468 and MCF-7 cells transfected with a mesenchymal reporter construct, and both MDA-MB-468 and PMC42-LA cells transfected with an epithelial reporter construct. These responsive subpopulations will enable measurements of epithelial and mesenchymal promoter states in the presence of the GFP-encoding GIPZ shRNA constructs, and are anticipated to be a useful reagent for others in this field (*e.g.* drug discovery). Indeed, in work outside the scope of this award, the screen has been used by collaborators with some 3,000 approved drugs (the so-called 're-purposing set" as well as a set of 140 kinase inhibitors, and has shown selective hist with ERK/MEK inhibitors and Src inhibitors. Similarly, a genome-wide screen for VIM induction in response to 23,000 full length cDNA (the ORFeome Open Reading Frame Collection) has been carried out and 74 highly significant hits have been found and will be followed up.

- PMC42-LA cells transduced with the boutique library, and sub-pools thereof, have been determined to have altered proportions of cells capable of forming mesenchymal colonies. Sub-pools of transduced cells with the highest proportion of cells capable of forming mesenchymal colonies have also been found to have a larger proportion of cells with low EPCAM surface expression. Consistent with this, cells with low EpCAM surface levels have a more mesenchymal phenotype. This is enabling selection of cells within the transduced sub-pools that are more mesenchymal and permit identification of which shRNA constructs are enriched for in these subpopulations.

- Although tumors grow very slowly, EpCAM-high and EpCAM-low populations have been sorted from the transduced PMC42-LA cells and grafted into the mammary fat pad of

SCID mice. Tumorigenicity, epithelial-mesenchymal modulation, and (eventually) metastastasis endpoints will be evaluated in these mice.

Reportable Outcomes

PRIMARY RESEARCH ARTICLES

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REVIEWS / BOOK CHAPTERS

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Kokkinos, M.I., Murthi, P., Wafai, R., THOMPSON E.W., Newgreen, D.F. Cadherins in the human placenta – epithelial-mesenchymal transition (EMT) and placental development. Placenta 31: 747-755 (2010)

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Gunasinghe, N.P.A.D., Wells, A., THOMPSON, E.W., Hugo H,J. Mesenchymal-epithelial transition (MET) as a mechanism for metastatic colonisation in breast cancer. Cancer Metastasis Reviews 2012 Jun 23. [Epub ahead of print]

CONFERENCES PRESENTATIONS

International Bone & Mineral Society – Cancer and Bone Society meeting, Chicago, IL, USA; Nov. 29 – Dec. 3, 2011. Erik Thompson. Epithelial Mesenchymal Plasticity and Breast Cancer Metastasis in Bone and Bone Marrow. (*International, Talk*)

Joint TuMIC - Metastasis Research Society - Champalimaud Foundation Conference 'New Concepts in Cancer Metastasis', Champalimaud Cancer Centre, Lisbon, Portugal; June 25 – 28, 2011. Erik Thompson..Epithelial Mesenchymal Plasticity and Breast Cancer Metastasis – Insights from Human Breast Cancer Cell Lines. Also Session Chair, Session 8. (*International, Talk*)

Keystone Symposium on Epithelial Plasticity and Epithelial to Mesenchymal Transition, Vancouver, B.C., Canada; Jan. 21-26, 2011. Erik Thompson. Epithelial Mesenchymal Plasticity in Human Breast Cancer Cell Lines: The Path Forward? (*International, Talk*)

Herrenhausen Symposium on Metastasis, Kloster Seeon, Seeon, Germany; October 8 – 11, 2012. Erik Thompson. Molecular Mechanisms of Metastasis Epithelial Mesenchymal Plasticity and Metastasis. (*International, Talk, Session Chair*)

20th Annual Meeting of the Japanese Association of Metastasis Research, Hiroshima, Japan; July 11-12, 2012. International Symposium Chair (*International, Chair*)

US-DOD Era of Hope, Orlando, Florida. August, 2011. Izhak Haviv, Tony Blick, Cletus Pinto, Mark Waltham, Erik Thompson. A Functional Genomic Screen for Tumorigenicity and Epithelial-Mesenchymal Transition *(International, Poster)*

102nd Annual AACR meeting, Orlando, FLA, USA; April 2 – 6, 2011. Honor Hugo, Bryce JW van Denderen, Eva Tomaskovic-Crook, Tony Blick, Dexing Huang, Cletus Pinto, Eliza Soo, Angels Fabra-Fres, Izhak Haviv, Gregory Goodall, Nicholas Wong, Leigh Ackland, Donald F. Newgreen, Mark Waltham, Erik W. Thompson. Coordinated Regulation of Mesenchymal Epithelial Transition in the PMC42-LA Breast Cancer Cell Line Variant. *(International, Poster)*

2012 Lorne Genome Conference, Lorne, Australia, February, 2011. Cletus Pinto, Tony Blick, Izhak Haviv, Mark Waltham, Erik Thompson. Understanding Epithelial Mesenchymal Plasticity in Breast Cancer – A Functional Genomics Approach (*National, Poster)*

ANZ BCTG 33rd Annual Participants' Scientific Meeting, Royal Pines Resort, Gold Coast, QLD; July 22-23, 2011. Erik Thompson. Epithelial Mesenchymal Plasticity – New Opportunities for Targeting Breast Cancer. (*National, Poster)*

The 16th International Colloquium on Lung and Airway Fibrosis, Busselton, WA; 30 October – 3 November, 2010. Erik Thompson. Epithelial Mesenchymal Plasticity and Pathogenesis: Breast Cancer as a Model Case. (*National, Poster)*

American Association for Cancer Research Annual Meeting, Chicago, IL, USA; 2012 March 31 - April 4. Erik Thompson. Epithelial mesenchymal plasticity in xenograft models of circulating and disseminated tumour cells from human breast cancer (*International, Talk*)

CONCLUSION

shRNA library transduction has enhanced tumorigenicity and modulated the PMC42-LA epithelial-mesenchymal properties of this cell, but how this has impacted on breast cancer cell metastasis in vivo (both PMC42-LA and MDA-MB-468 cells) is yet to be established in our final phase of work.